REMARKS

Amendments

The foregoing amendments merely cancel non-limiting preamble verbiage and to change their dependency from cancelled claim 1 to claim 11. Claim 65 is canceled because it no longer has antecedent basis. These amendments introduce no new matter.

35USC102(a)

The composition of claim 11 requires a *nonvirulent* bacterium comprising a first gene encoding a *nonsecreted* foreign functional *cytolysin* operably linked to a heterologous promoter which expresses the cytolysin in the bacterium, and a second gene encoding a different foreign antigenic agent. The cited art does not meet at least three of these limitations.

First, the bacterium of Dietrich is a Listeria monocytogenes only *attenuated* for virulence - it is not avirulent; second, the foreign lysin gene of Dietrich et al. is *secreted*, and necessarily so; and third, it is a peptidase *endolysin*, not a cytolysin.

Dietrich's method uses a Listeria monocytogenes bacterium having attenuated virulence and expressing a bacteriophage lysis gene under the L. monocytogenes *actA* promoter, which is activated in the cytosol of the infected cell; Dietrich, p.181, col.2, lines 17-18. Note that Dietrich's bacteria are specifically designed to escape to the cytosol of the infected host cell where the foreign lysis gene can be activated; Dietrich, p.181, col.1, line 24; p.184, col.2, lines 7-9. However, Dietrich's expressed lysis gene does not lyse any cellular membranes - in fact it is secreted through the membrane of the bacteria and attacks a component of the surrounding bacterial wall. More particularly, the lysin used by Dietrich et al., PLY118, is a peptidase endolysin - it does not lyse cell membranes so it is not a cytolysin. PLY118 specifically digests a component of the cell *wall* of Listeria; see Dietrich et al. p.182, col.1, line 1, where Dietrich cites Loessner et al., 1995, Mol Microbiol 16, 1231-41, abstract enclosed. Loessner explains that PLY118 is a cell wall lytic enzyme which specifically cleaves between the L-alanine and D-glutamate residues of listerial peptidoglycan.

Published with the Dietrich et al. paper was an analysis written by the present inventors, Higgins and Portnoy, 1998, Nature Biotechnol 16, 138-139, enclosed, wherein Figure 1

illustrates Dietrich's method; see also p.139, col.3, explaining the difference between strains of attenuated virulence, as in Dietrich et al., and avirulent strains. The cited Dietrich report neither describes nor suggests the claimed invention: it describes a different, incompatible protocol requiring different, inexchangeable reagents and operates under a different mechanism.

35USC112, second paragraph

The preamble of claims 40-65 have been amended to correspond to the claimed method.

35USC112, first paragraph (enablement)

The enablement requirement of 35USC112, first paragraph provides that the specification enable one of ordinary skill in the art to practice the invention without undue experimentation. As amended, claims 53-64 are directed to a method of introducing a foreign therapeutic agent into a eukaryotic cell by the step of: contacting the cell with the bacterium of claim 11 under conditions whereby the agent enters the cell. The bacterium of claim 11 is a nonvirulent bacterium comprising a first gene encoding a nonsecreted foreign functional cytolysin operably linked to a heterologous promoter which expresses the cytolysin in the bacterium, and a second gene encoding a different foreign antigenic agent. The issue then, is whether, given the teachings of the specification, one skilled in the art would require undue experimentation to introduce a foreign therapeutic agent into a eukaryotic cell by contacting the cell with the bacterium of claim 11 under conditions whereby the agent enters the cell.

The specification generally teaches the introduction of a variety of foreign agents for a variety of purposes (p.4, line 11 - p.5, line 26), including therapeutic agents. The specification generally teaches a variety of effective routes of administration depending on the nature of the foreign agent (p.7, line 5 - p.8, line 5). In addition, the specification provides numerous demonstrative examples of the method as applied to numerous foreign therapeutic agents (p.8, line 6 - p.10, line 11).

The only distinguishing feature of the presently rejected claims is that a foreign *therapeutic* agent is recited instead of a foreign *antigenic* agent. This involves no more than substituting one foreign agent for another in an empirically simple method. In fact, in some

embodiments, an antigenic agent will suffice as a therapeutic agent; therapeutic agents include prophylactics such as immunizations (p.4, lines 13-14). In addition, a wide variety of alternative agents are described and exemplified (p.9-10, col.3 of Table 1). The application provides more than sufficient teaching to enable one of ordinary skill in this art to practice the claimed method without undue experimentation.

The Examiner is invited to call the undersigned if she would like to amend the claims to clarify the foregoing or seeks further clarification of the claim language.

Applicants hereby petition for any necessary extension of time pursuant to 37 CFR 1.136(a). The Commissioner is hereby authorized to charge any fees or credit any overcharges relating to this communication to our Deposit Account No. 19-0750 (order no. B98-039-2).

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

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encl. Higgens and Portnoy, 1998, Nature Biotechnol 16, 138-139;
Loessner et al., 1995, Mol Microbiol 16, 1231-41 (abstract)

1: *Mol Microbiol* 1995 Jun;16(6):1231-41

Related Articles, Books, Protein, Nucleotid

Heterogeneous endolysins in Listeria monocytogenes bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes.

Loessner MJ, Wendlinger G, Scherer S

Institut fur Mikrobiologie, Technische Universitat Munchen, Freising, Germany.

Listeria monocytogenes bacteriophages A118, A500 and A511 are members of three distinct phage groups with characteristic host ranges. Their endolysin (ply) genes were cloned and expressed in Escherichia coli as demonstrated by the conferred lytic phenotype when colonies of recombinant cells were overlaid with a lawn of Listeria cells. The nucleotide sequences of the cloned DNA fragments were determined and the individual enzymes (PLY118, 30.8 kDa; PLY500, 33.4 kDa; PLY511, 36.5 kDa) were shown to have varying degrees o homology within their N-terminal or C-terminal domains. Transcriptional analysis revealed them to be 'late' genes with transcription beginning 15-20 min post-infection. The enzymes were overexpressed and partially purified and their individual specificities examined. When applied exogenously, the lysins induced rapid lysis of Listeria strains from all species but generally did not affect other bacteria. Using hydrolysis of purified listerial cell walls, PLY511 was characterized as an N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) and shows homology in its N-terminal domain to other enzymes of this type. In contrast, PLY118 and PLY500 were shown to represent a new class of cell wall lytic enzymes which cleave between the L-alanine and D-glutamate residues of listerial peptidoglycan; these were designated as L-alanoyl-D-glutamate peptidases. These two enzymes share homology in the N-terminal domain which we propose determines hydrolytic specificity. Highly conserved holin (hol) gene sequences are present upstream of ply118 and ply500. They encode proteins of structural similarity to the product of phage lambda gene S, and are predicted to be membrane proteins which form pores to allow access of the lysins to their peptidoglycan substrates. This arrangement of conserved holin genes with downstream lysin genes among the siphoviral lysis cassettes explains why the cytoplasmic endolysins alone are not lethal, since they require a specific transport function across the cell membrane.

PMID: 8577256

B96-039-1

morphological transformation in vitro correlates well with carcinogenesis, but can be detected 2–8 months earlier[14]. As carcinogens they chose the aromatic polycyclic hydrocarbons, benzopyrene, methylcholanthrene, and dimethylbenzanthracene, which are not genotoxic in most test systems, including CHE cells in vitro[15], and colcemid, which is not even mutagenic after treatment with liver enzymes[16].

Their results were unambiguous: ". . .38 out of 38 transformed colonies and subcolonies of carcinogen-treated CHE cells, 2 out of 2 transformed colonies of colcemid-treated CHE cells, and 4 out of 4 spontaneously transformed colonies of CHE cells were 50 to 100% aneuploid. Because 39 of these 44 transformed colonies contained more than 50% of aneuploid cells, the aneuploidy must have originated in the same cell from which the transformed colony originated."[14] When tested in an animal model, 4 out of 4 transformed colonies were strongly tumorigenic. All of the transformants, although maintaining an aneuploid state, were nonetheless genetically highly unstable despite their clonal origin, reminiscent of the in vivo situation where a "confusing plethora" of aneuploidies is apparent even in the clonal stem line cells of primary tumors[17]. This result opens the possibility of analyzing such transformants for the long-sought "specific, causative aneuploidy," although as discussed below, models of aneuploid promoted cancer do not require specific chromosome imbalances.

Much work remains to be done before the aneuploidy hypothesis is fully rejuvenated. Many more colonies of transformed cells need to be examined. The issue of whether, after single-cell cloning, all transformed cells from colonies with diploid cells are aneuploid, needs to be answered. More colonies need to be tested for their tumorigenicity, and the experiments repeated with genotoxic carcinogens like methylnitroso urea, which the gene mutation hypothesis clearly predicts should produce diploid transformants. The present results are nonetheless compelling enough to provoke both practical and theoretical interest.

In cancer diagnosis, for example, one can imagine tests for aneuploidy instead of point-mutated oncogenes, and in cancer prevention, the ability to induce aneuploidy in culture might be used to assess a substance's carcinogenic potential. This new incarnation of the aneuploidy hypothesis should also appeal to theoreticians, as the quantitative and global aspects of aneuploidy make its effects ideally suited to a treatment using the conceptual approaches and mathematical tools of metabolic control theory[18], which has succeeded in replacing the over-specified idea of the "rate limiting enzyme" with the much more powerful one of "distributed control," a situation analogous to the oncogene and aneuploidy.

A recent paper in *Science* by Lin Zhang and colleagues[19] provides unanticipated support for such a view of carcinogenesis. Using a functional genomic approach, serial analysis of gene expression (SAGE), the authors compared differences in gene expression between normal and colon cancer cells. Reasoning that "the genes exhibiting the greatest differences in expression are likely to be the most biologically important," they limited their analysis to the approximately 300 genes showing a tenfold or greater difference in expression, out of a total of 5000 differentially expressed genes. The results were surprising. In the author's words, "most of the transcripts could not have been predicted to be differentially expressed." More pointedly, not a single oncogene or tumor suppressor gene was reported to show the expected difference in expression. In fact, "two widely studied oncogenes, *c-fos* and *c-erb3*, were expressed at much higher levels in normal colon epithelium than in (colorectal) cancers", and the grandfather of oncogenes, *ras*, was "expressed at very low levels in all tissues examined and no significant changes in cancer cells compared with normal cells were detected" (personal correspondence from L. Zhang and B. Vogelstein to P. Duesberg, November 3, 1997), although transformation by *ras* in vitro requires more than a 100-fold increased expression[20].

While difficult to reconcile with the gene mutation hypothesis, these results do make sense from the perspective of aneuploidy and metabolic control theory, in which the fraction of the wild-type genome that undergoes a change in expression, rather than changes in the masses of particular mRNAs, becomes the defining parameter. In this view, the radically perturbed phenotype of the cancer cell does not depend on the effects of relatively large differences in the expression of one or more mutant oncogenes or tumor suppressor genes. Instead, it results from an altered "genomic flux" that is an aggregate effect of smaller changes in the expression of thousands of genes contributed by the aneuploidy.

With the enormous amount of DNA sequencing, comparison and functional analysis currently underway, molecular geneticists are accumulating a sufficiently extensive dataset (comparable to the one available to metabolic control theorists) to make the expectation of fashioning a dynamic theory of the genome realistic. Such a theory would truly explain the ontology of robust, aberrant phenotypes like cancer, and within it the oncochromosome may come to supplant the oncogene as the primary experimental focus.

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Bacterial delivery of DNA evolves

Darren E. Higgins and Daniel A. Portnoy

The capacity to deliver macromolecules (i.e., antigens and DNA) to specific tissues, cells, or cellular compartments remains one of the biggest challenges facing the biotechnology community. One approach is to exploit mechanisms used by microbial pathogens—mechanisms that have coevolved with their mammalian hosts for millions of years. Intracellular pathogens, for example, have evolved specific mechanisms of entry and growth within host cells. Some intracellular pathogens reside in vacuoles, whereas others escape from a vacuole and enter the host cytosol. The molecular and cell biology of these processes has received considerable attention in recent years, and the molecular mechanisms involved are currently being elucidated[1]. In this issue, Dietrich et al.[2] have engineered the intracellular pathogen *Listeria*

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monocytogenes to deliver plasmid DNA into the cytosol of macrophages. This has implications for the specific delivery of DNA and other macromolecules to macrophages and other cell types in vivo.

L. monocytogenes has been used for decades as a model system to study aspects of the cellular immune system[1]. In this model, antibody plays no measurable role and immunity is mediated by $CD8^+$ T cells. During the past few years, the cell biology of infection has also been closely scrutinized, providing us with a cell biological explanation for the requirement of cell-mediated immunity in *L. monocytogenes* infections[1]. Subsequent to phagocytosis, these bacteria mediate lysis of the vacuole and enter the cytosol where rapid growth ensues. By exploiting the host system of actin-based motility, *L. monocycogenes* is able to spread from cell-to-cell without contacting the extracellular milieu. During intracellular growth, however, secreted bacterial proteins are delivered into the major histocompatibility class I pathway of antigen processing, and derived peptides are presented on the cell surface to $CD8^+$ T cells[4].

Two bacterial products largely responsible for mediating the cell biology of infection have been well characterized. The first is a secreted pore-forming protein called listeriolysin O (LLO)[5]. LLO is the primary determinant of pathogenicity responsible for lysis of the vacuole and is a member of a family of pore-forming proteins including streptolysin O from *Streptococcus pyogenes* and perfringolysin O from *Clostridium perfringens*. The capacity of *L. monocycogenes* to exploit the host system of actin-based motility relies on a second characterized bacterial surface protein, ActA[2]. ActA is responsible for polymerizing host-cell actin and its expression is induced within the cytosol of the host.

The growth of *L. monocytogenes* in the host cytosol has made it an attractive candidate as a live vaccine vector for the induction of cell-mediated immunity to foreign antigens. Indeed, during the past few years, recombinant *L. monocytogenes* has been used successfully for expression of antigens and induction of $CD8^+$ T cells to influenza virus, lymphocytic choriomeningitis virus, human immunodeficiency virus, and tumors[6–9]. An alternative approach for the delivery of antigens has been to use mixtures of soluble protein and purified LLO, either alone or encapsulated into pH-sensitive liposomes[10,11]. Both approaches have shown promising results.

Dietrich et al. have now used *L. monocytogenes* to introduce plasmid DNA into macrophages. They developed an attenuated suicide strain lacking the *actA* gene and designed to lyse inside of the host cytosol by expressing a *Listeria*-specific phage lysin from the *actA* promoter. Using expression of green fluorescent protein as a readout, they showed that 0.03% of a macrophage cell line infected with approximately one bacterium per cell expressed plasmid DNA. This increased to >0.2% when a wild-type *actA*$^+$ strain expressing the phage lysin was used to deliver DNA.

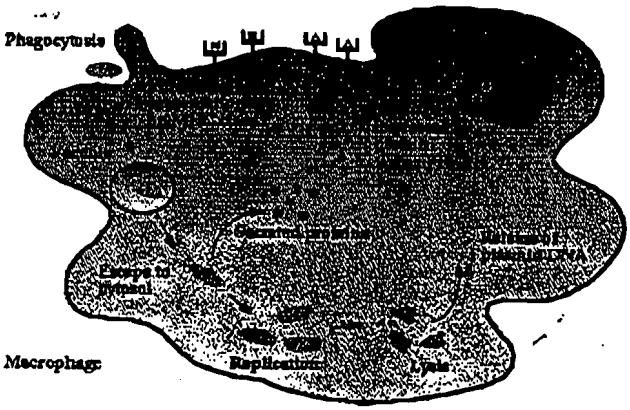

Figure 1. Model for *Listeria monocytogenes*–based delivery of protein and plasmid DNA. Following phagocytosis and escape from the vacuole, foreign proteins secreted by recombinant *L. monocytogenes* can be processed and presented on the surface for recognition by $CD8^+$ T cells. Alternatively, attenuated *L. monocytogenes* expressing a *Listeria*-specific phage lysin replicate briefly in the cytosol, and expression of the phage lysin is induced. Phage lysin-mediated lysis of the bacteria allows the release of antigen-encoding plasmid DNA.

Although the frequency of transfection is low compared with other methods, such as electroporation or retroviral vectors, the lack of a need to produce multiple vector constructs for delivery and the potential to deliver DNA in vivo make the use of attenuated *L. monocytogenes* a very attractive alternative. The authors further show that infection of bone marrow-derived macrophages by *L. monocytogenes*-containing plasmid DNA encoding ovalbumin results in the presentation of antigen to ovalbumin-specific $CD8^+$ T cells.

Whereas this is the first use of *L. monocytogenes* to deliver DNA, two other groups have already shown that *Shigella flexneri* can deliver DNA to cultured mammalian cells[12,13]. In both cases, strains impaired in cell-wall synthesis that required diaminopimelic acid (DAP) for growth were employed. In the absence of DAP, the bacteria lyse in the cytosol releasing plasmid DNA. Work in our group has generated a D-alanine–requiring strain of *L. monocytogenes*, but found that this strain was so attenuated that without D-alanine, it was highly defective even in gaining entry to the cytosol (R.J. Thompson, H.G.A. Bouwer, D.A. Portnoy, and F.R. Frankel, unpublished data). In contrast, the attenuated suicide strain described by Dietrich et al. appears to enter the cytosol normally and lyse after a few generations. As this strain also lacks the wild-type *actA* gene, it is severely attenuated for virulence. Recently, an attenuated *Salmonella typhimurium* strain carrying antigen-encoding plasma DNA has been used in vivo to induce both humoral and cellular immune responses[14]. In this case, the *S. typhimurium* was administered orally and the kinetics of the immune responses suggested that host cell expression of plasmid DNA, not bacterial expressed protein, was responsible for immunization.

What is the best direction for the future? Should we use live, attenuated bacteria like that used in this study, or should we use purified bacterial components such as LLO encapsulated in liposomes? Certainly, bacteria are less expensive, but there is always a potential risk to the host. A third approach is to engineer avirulent *Escherichia coli* K12 for delivery of antigen and DNA, an approach that is currently being investigated by several laboratories. It has been shown that DAP-negative *E. coli* K12 containing a plasmid from *S. flexneri* conferring an invasive phenotype can be used to deliver DNA to cultured mammalian cells[15]. In addition to the decreased hazards associated with engineered avirulent *E. coli*, the high level of plasmid copies attainable and ease of genetic manipulation are distinct advantages. Recently, advances in the use of another approach—naked DNA vaccinations—have suggested an alternative to the use of carriers for the delivery of DNA to cells[15]. However, the benefit of using invasive bacteria that have evolved to invade a variety of cell types is a viable approach to be further explored for the delivery of DNA and protein both in vitro and in vivo.

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L9 ANSWER 9 OF 35 MEDLINE
ACCESSION NUMBER: 95053755 MEDLINE
DOCUMENT NUMBER: 95053755
TITLE: Delivery of a viral antigen to the class I processing and presentation pathway by Listeria monocytogenes.
AUTHOR: Ikonomidis G; Paterson Y; Kos F J; **Portnoy D A**
CORPORATE SOURCE: Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia 19104-6076.
CONTRACT NUMBER: AI-27655 (NIAID)
GM-31841 (NIGMS)
SOURCE: JOURNAL OF EXPERIMENTAL MEDICINE, (1994 Dec 1) 180 (6) 2209-18.
Journal code: I2V. ISSN: 0022-1007.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199502

AB Listeria monocytogenes is a facultative intracellular pathogen that grows in the cytoplasm of infected host cells. We examined the capacity of L. monocytogenes to introduce influenza nucleoprotein (NP) into the class I pathway of antigen presentation both in vitro and in vivo. Recombinant L. monocytogenes secreting a fusion of **listeriolysin** O and NP (LLO-NP) targeted infected cells for lysis by NP-specific class I-restricted cytotoxic T cells. Antigen presentation occurred in the context of three different class I haplotypes in vitro. A hemolysin-negative L. monocytogenes strain expressing LLO-NP was able to present in a class II-restricted manner. However, it failed to target infected cells for lysis by CD8+ T cells, indicating that hemolysin-dependent bacterial escape from the vacuole is necessary for class I presentation in vitro. Immunization of mice with a recombinant L. monocytogenes strain that stably expressed and secreted LLO-NP induced NP-specific CD8+ cytotoxic T lymphocytes. These studies have implications for the use of L. monocytogenes to deliver potentially any antigen to the class I pathway in vivo.

L9 ANSWER 6 OF 35 MEDLINE
ACCESSION NUMBER: 96205886 MEDLINE
DOCUMENT NUMBER: 96205886
TITLE: Delivery of macromolecules into cytosol using liposomes containing hemolysin from Listeria monocytogenes.
AUTHOR: Lee K D; Oh Y K; **Portnoy D A**; Swanson J A
CORPORATE SOURCE: Department of Cell Biology, Harvard Medical School, Boston,
Massachusetts 02115, USA.
SOURCE: JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Mar 29) 271 (13) 7249-52.
Journal code: HIV. ISSN: 0021-9258.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199609

AB The cytosolic space of cells is an important but relatively inaccessible target for the delivery of therapeutic macromolecules. Here we describe the efficient delivery of macromolecules into the cytosolic space of macrophages from liposomes that contain **listeriolysin** O (LLO), the hemolytic protein of Listeria monocytogenes that normally mediates bacterial passage from phagosomes into cytosol. LLO was purified and encapsulated inside pH-sensitive liposomes, along with other molecules to be delivered. When internalized by bone marrow-derived macrophages, these liposomes rapidly released encapsulated fluorescent dye, first into endosomes and then into the cytosol, without measurably harming the cells.

Furthermore, these liposomes efficiently delivered encapsulated ovalbumin to the cytosolic pathway of antigen processing and presentation, as measured by the major histocompatibility complex (MHC) class I-restricted presentation of peptides derived from ovalbumin. Delivery was significantly better than that obtained by other currently available liposome formulations. LLO-containing liposomes should therefore provide an efficient vehicle for delivery of antigens or therapeutic molecules in vivo.

9 ANSWER 4 OF 35 MEDLINE
ACCESSION NUMBER: 1998148494 MEDLINE
DOCUMENT NUMBER: 98148494
TITLE: Bacterial delivery of DNA evolves [news; comment].
COMMENT: Comment on: Nat Biotechnol 1998 Feb;16(2):181-5
AUTHOR: Higgins D E; **Portnoy D A**
SOURCE: NATURE BIOTECHNOLOGY, (1998 Feb) 16 (2) 138-9.
Journal code: CQ3. ISSN: 1087-0156.
PUB. COUNTRY: United States
Commentary
News Announcement
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199806
ENTRY WEEK: 199806

TITLE: Delivery of protein to the cytosol of macrophages using Escherichia coli K-12.
AUTHOR: Higgins D E; Shastri N; **Portnoy D A**
CORPORATE SOURCE: Department of Molecular and Cell Biology, University of California, Berkeley 94720, USA.
CONTRACT NUMBER: AI-27655 (NIAID)
SOURCE: MOLECULAR MICROBIOLOGY, (1999 Mar) 31 (6) 1631-41.
Journal code: MOM. ISSN: 0950-382X.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199909
ENTRY WEEK: 19990902
AB **Listeriolysin** O (LLO) is an essential determinant of pathogenicity whose natural biological role is to mediate lysis of Listeria monocytogenes containing phagosomes. In this study, we report that Escherichia coli expressing cytoplasmic recombinant LLO can efficiently deliver co-expressed proteins to the cytosol of macrophages. We propose a model in which subsequent or concomitant to phagocytosis the E. coli are killed and degraded within phagosomes causing the release of LLO and target proteins from the bacteria. LLO acts by forming large pores in the phagosomal membrane, thus releasing the target protein into the cytosol. Delivery was shown to be rapid, within minutes after phagocytosis. Using this method, a large enzymatically active protein was delivered to the cytosol. Furthermore, we demonstrated that the E. coli/LLO system is very efficient for delivery of ovalbumin (OVA) to the major histocompatibility (MHC) class I pathway for antigen processing and presentation, greater than 4 logs compared with E. coli expressing OVA alone. Moreover, the time required for processing and presentation of an OVA-derived peptide was similar to that previously reported when purified OVA was introduced directly into the cytosol by other methods. Using this system, potentially large amounts of any protein that can be expressed in E. coli can be delivered to the cytosol without protein purification. The potential use of this system for the delivery of antigenic protein in vivo and the delivery of D